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(54) Title: METHOD FOR GENERATING REPLACEMENT CELLS AND/OR TISSUES

(57) Abstract: Disclosed are methods of isolating differentiated cells or adult stem cells from pluripotent cells and particularly ICM cells, by exposing such pluripotent cells to environmental cues in order to encourage development along a certain path.

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Method for Generating Replacement Cells and/or Tissues

Related Applications

[0001] This application claims benefit of priority to U.S. Serial No. 60/274,216 filed on March 9, 2001 and of which are incorporated by reference in their entirety herein.

Field of Invention

[0002] The present invention is concerned with engineering replacement cells and tissues that may be used for transplantation into patients in need of such tissues. In particular, the present invention provides a means for producing isogenic replacement tissues using nuclear transfer, without requiring *in vitro* isolation and differentiation of embryonic stem cells.

Background of the Invention

[0003] Embryonic stem (ES) cell technology is one of the few known means for generating large numbers of replacement cell types from pre-implantation stage embryos. Nuclear transfer technology in particular enables the isolation of ES and inner cell mass (ICM) cells from a differentiated somatic cell nuclear donor. This technology provides great promise for engineering cells and tissues for human transplantation, because such tissues can be designed using the patient's own cells as the source of nuclear DNA, thereby avoiding the problems of transplant rejection and graft versus host reactions associated with transplanting allogeneic and xenogeneic tissues.

[0004] Despite the promise of this technology, many years of research may still be required before biologists understand how to direct ES cells *in vitro* along a desired line of development in order to generate replacement cells and tissues for human patients. Furthermore, it may be ethically and legally problematic to implant human and/or chimeric human-animal embryos into animals, or to implant human and/or chimeric human-animal ICMs/ES cells into animals that may have the capacity to generate embryos and/or teratomas. Thus, there is an immediate need for methods that enable isolation of

differentiated cells and tissues from ES cells or other pluripotent stem cells that do not require a knowledge of the developmental signals that guide embryonic development, and that do not raise ethical and legal dilemmas with regard to human patients.

[0005] In this regard, researchers have shown using an *in utero* xenotransplantation approach that neural progenitor cells from mice differentiate into cells having glial-like features after injection into the rat forebrain ventricle. See Winkler et al. (June 1998) Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain, Mol. Cell. Neurosci. 11(3): 99-116. Similarly, human neural precursor cells that had been expanded *in vitro* were shown to develop into neurons in a site-specific manner after being transplanted into either an adult or neonatal rat brain. See Fricker et al. (July 1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain, J. Neurosci. 19(14): 5990-6005; see also Rosser et al. (July 2000) The morphological development of neurons derived from EGF- and FGF-2-driven human CNS precursors depends on their site of integration in the neonatal rat brain, Eur. J. Neurosci. 12(7): 2405-13. In these studies, the resulting neuron cells were not purified but rather were traced by mouse-specific and human-specific markers. Nevertheless, these results suggest that neural progenitor cells respond to host-derived environmental signals that direct their differentiation along multiple phenotypic pathways.

[0006] Researchers from the University of South Florida have recently extended this premise to an *in vitro* setting by showing that it is possible to induce human or mouse bone marrow stromal cells (BMSC), which normally give rise to bone, cartilage, and mesenchymal cells, to differentiate into neuron-like cells by culturing them in the presence of rat fetal mesencephalic or striatal cells. See Sanches-Ramos et al (August 2000) Adult bone marrow stromal cells differentiate into neural cells *in vitro*, Exp. Neurol. 164(2): 247-56. Thus, it may be possible to mimic the environmental signals that induce pluripotent cells to differentiate along a given pathway *in vitro* merely by exposing

pluripotent cells to differentiated cells. However, it is not clear how useful cellular engraftment will be in repairing damaged tissues absent tissue engineering, therefore, differentiated cells derived *in vitro* still have limited utility.

[0007] Furthermore, in order to employ cells that have been environmentally induced to differentiate along a certain developmental path, one must have a means of isolating or purifying such cells away from the inducing cells. This is particularly true where human cells are induced to differentiate in the presence of rat cells or other animal cells that might harbor viruses or other infectious agents that could potentially present a danger to human patients receiving such replacement cells. In this regard, Klug et al have described an approach for isolating relatively pure cardiomyocytes from differentiating murine embryonic stem (ES) cells whereby ES cells are stably transfected with a fusion gene consisting of a selectable marker, aminoglycoside phosphotransferase, linked to the alpha-cardiac myosin heavy chain promoter. See Klug et al (July 1996) Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intercardiac grafts, J. Clin. Invest. 98(1): 216-24; see also McWhir et al (Oct 1996) Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a nonpermissive genetic background, Nat. Genet. 14(2): 223-6 (using a similar technique to select against differentiated cells). The aminoglycoside phosphotransferase-transfected cells in Klug were differentiated *in vitro* and subjected to G418 selection, and the resulting cardiomyocyte cultures were shown to be highly pure (>99%). Further, stable ES-cell derived cardiomyocyte grafts were observed in the hearts of adult mice seven weeks after implantation. However, as noted by Klug in this report, cellular engraftment has questionable utility in effecting myocardial repair.

[0008] Thus, there is still a need for methods which allow the production and isolation of differentiated cells, adult stem cells and tissues for replacement therapy. Moreover, it would be advantageous to design such replacement cells to genetically match the patient to avoid rejection of the cells and tissues and other harmful immune responses following transplantation into the patient.